

# BLOOD

*The Journal of  
The American Society of  
Hematology*

VOL 78, NO 4

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AUGUST 15, 1991

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Applicants: David J. Pinsky  
U.S. Serial No.: 09/374,586  
Filed: August 13, 1999  
Group Art Unit: 1633

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All manuscripts submitted to BLOOD and subsequently accepted for publication will be assessed a \$50.00 charge per published page. This charge is necessary to offset the increasing costs of publication.

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# Inhibition of Human Platelet Reactivity by Endothelium-Derived Relaxing Factor From Human Umbilical Vein Endothelial Cells in Suspension: Blockade of Aggregation and Secretion by an Aspirin-Insensitive Mechanism

By M. Johan Broekman, Ana M. Eiroa, and Aaron J. Marcus

To determine a role for endothelium-derived relaxing factor/nitric oxide (EDRF/NO) in regulation of human platelet reactivity by human endothelial cells (EC), we studied combined suspensions of human umbilical vein endothelial cells (HUVEC, passage 2 through 3) and washed human platelets. Confluent HUVEC monolayers were treated with aspirin (1 mmol/L) to prevent prostacyclin (PGI<sub>2</sub>) formation, washed, and harvested. Aspirin-treated platelets alone ( $58 \times 10^4$ ) were fully aggregated by thrombin at 0.05 U/mL or more. In the presence of  $10^6$  HUVEC, however, platelet serotonin release and aggregation in response to thrombin at doses as high as 0.5 U/mL were blocked. We demonstrated for the first time that inhibition of aggregation and serotonin release, due to EDRF/NO, occurred in parallel. HUVEC-dependent inhibition of platelet responsiveness was enhanced by superoxide dismutase (SOD) and reversed by

hemoglobin. The inhibitory effect was also reversed by preincubation of HUVEC with *N*<sup>G</sup>-monomethyl-L-arginine (NMA) or *N*<sup>G</sup>-nitro-L-arginine (NNA) through competitive blockade of arginine metabolism. Pretreatment of platelets with methylene blue indicated that EC-dependent inhibition of platelet reactivity occurred through activation of platelet soluble guanylate cyclase. When platelets and HUVEC were separated by a permeable membrane and both cells were stimulated by thrombin, platelets remained unresponsive. This indicated that inhibition was induced by a fluid-phase mediator, independent of direct cell-cell contact. These data demonstrate that EDRF/NO formation from L-arginine by human EC plays an important role as an aspirin-insensitive fluid-phase inhibitor of human platelet reactivity.

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## MATERIALS AND METHODS

**Materials.** Materials used were HEPES-buffered saline (HBS) (in mmol/L): NaCl 137.3, KCl 3.64, HEPES 10, glucose 11, pH 7.4. Complete medium was medium 199 with Earle's salts and 25 mmol/L HEPES (GIBCO, Grand Island, NY) supplemented with 20% human serum, 2 mmol/L L-glutamine (Sigma, St Louis, MO), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma). Collagenase/EDTA consisted of equal volumes collagenase (type 1, 200 U/mL; Worthington, Freehold, NJ) in HBS and 0.02% EDTA/0.5% bovine serum albumin (BSA; Sigma, A-9647) in HBS. Incubation buffer (in mmol/L) was KCl 4.2, MgSO<sub>4</sub> 0.5, NaCl 135.5, Na<sub>2</sub>HPO<sub>4</sub> 6.5, NaH<sub>2</sub>PO<sub>4</sub> 1.5, glucose 5.6, pH 7.3.<sup>20</sup> Tris-citrate buffer (in mmol/L) was Tris 63, NaCl 95, KCl 5, citric acid 12, pH 6.5.<sup>23-27</sup> ACD anticoagulant (in mmol/L) was 75 trisodium citrate, 38 citric acid, and 135 glucose.<sup>27</sup>

Human serum was prepared from 280 mL whole blood collected by free flow into sterile 50-mL plastic centrifuge tubes. To obtain serum, whole blood was clotted by incubation (37°C, 2 hours), centrifuged (2,000g, 20 minutes, 22°C), sterilized by filtration (0.45 µm followed by 0.2-µm pore size), and stored in 25-mL aliquots at -70°C.

Gelatin was obtained from Baker (Phillipsburg, NJ). Human hemoglobin (Sigma H-7379) was dissolved in water (65 mg/mL), reduced with sodium hydrosulfite (10-fold molar excess, 10 minutes, 22°C), dialyzed against 2 L water (4°C, 2 hours), and stored

**E**NDOTHELIAL CELLS (EC) control platelet responsiveness to agonists by several mechanisms. Stimulated EC synthesize prostacyclin (PGI<sub>2</sub>), an antiaggregatory eicosanoid.<sup>1,2</sup> Activated platelets can initiate synthesis and release of PGI<sub>2</sub> from human umbilical vein endothelial cells (HUVEC).<sup>3</sup> This phenomenon is now known as transcellular metabolism.<sup>4,5</sup> Reactions by which one cell uses precursors and intermediates from another for production of increased quantities of eicosanoids, or new metabolites with different functions,<sup>4,8</sup> may explain previously unanswered clinical questions in vascular disease.<sup>9,10</sup>

Two additional pathways by which EC may affect cells in the local environment have been proposed. One is catabolism of ADP by EC ecto-ADPases<sup>11-14</sup>; the other is formation of endothelium-derived relaxing factor (EDRF).<sup>15-17</sup> In contrast to PGI<sub>2</sub>, these two pathways are aspirin-insensitive. In vitro experiments have demonstrated that responses of acetylsalicylic acid (ASA)-treated platelets to agonists such as thrombin remain inhibited in the presence of aspirin-treated EC.

Although aspirin treatment for prophylaxis and management of vascular disease has been promising in clinical trials, more than 65% of participants were not protected by aspirin (ASA).<sup>18</sup> Furthermore, ASA does not reduce the severity of cardiovascular events when they do occur.<sup>19</sup> Therefore, information on possible ASA-insensitive approaches to complement aspirin therapy is of clinical relevance.

Thus far, one group of investigators has reported inhibitory effects on platelet aggregation responses by EDRF formation from HUVEC cultured on microcarrier beads.<sup>20</sup> We recently devised two in vitro systems to demonstrate EDRF generation by HUVEC in suspension and its effects on human platelet secretion as well as aggregation.<sup>21,22</sup> The present studies establish for the first time that human platelet serotonin secretion as well as aggregation in response to thrombin are blocked by HUVEC through formation of EDRF/nitric oxide, a fluid-phase ASA-insensitive autacoid.

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Submitted October 19, 1990; accepted April 19, 1991.

Supported in part by grants from the Department of Veterans Affairs, and from the National Institutes of Health HL-29034 (M.J.B.), and HL-18828, HL-46403, and HL-47073 (A.J.M. and M.J.B.).

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0006-4971/91/7804-0015\$3.00/0

( $\leq 1$  month,  $-70^{\circ}\text{C}$ ). L-Arginine,  $N^{\omega}$ -nitro-L-arginine (NNA), bovine erythrocyte superoxide dismutase (SOD), and methylene blue were from Sigma.  $^{14}\text{C}$ -Serotonin (5-HT, 54 mCi/mmol) was from Amersham, Arlington Heights, IL. Sodium arachidonate was from Nu-Check-Prep, Elysian, MN. Aquasol-2 was from NEN-DuPont, Boston, MA.  $N^{\omega}$ -monomethyl-L-arginine acetate (NMA) was a gift from Dr S. Moncada, Wellcome Research Laboratories, Beckenham, Kent, UK and Dr S. Gross, Cornell University Medical College or was purchased from Calbiochem (La Jolla, CA).  $N^{\omega}$ -monomethyl-D-arginine was from Drs Moncada and Gross. Tissue culture flasks and polypropylene 50-mL centrifuge tubes were from Falcon, Becton Dickinson (Lincoln Park, NJ). Biosilon polystyrene microcarrier beads<sup>20</sup> were from Nunc, Newbury Park, CA, and Millicell-HA culture plate inserts (12 mm, 0.45- $\mu\text{m}$  pore size) were from Millipore, Bedford, MA.

**Isolation of HUVEC.** HUVEC were isolated from fresh umbilical cords and cultured by the method of Jaffe et al.,<sup>3,28</sup> with slight modifications. Tissue culture flasks were coated with 0.2% gelatin. Primary cultures were grown in 25-cm<sup>2</sup> flasks in complete medium. Confluent monolayers with the typical cobblestone morphology of EC were passaged (1:3 split) with collagenase/EDTA and subcultured in 75-cm<sup>2</sup> flasks in complete medium. Cells reached confluence in about 3 days. Usually, EC from two to four cords were combined at first passage and further subcultured as a mixed population.

Eighteen hours before experiments, HUVEC, at or near confluency, were supplied with fresh complete medium. In preliminary experiments, extended subculturing, as well as other culture conditions (absence or presence of EC growth supplement and heparin, fungizone) had minimal effects. HUVEC from up to passage 8 inhibited platelet reactivity through a mediator with EDRF/NO characteristics (data not shown); however, the data we present were obtained with HUVEC of passages 2 and 3. To isolate EC, confluent monolayers were washed twice with prewarmed (37°C) HBS. HUVEC cyclooxygenase was inhibited with 1 mmol/L ASA, dissolved in medium 199 (30 minutes, 37°C), after which monolayers were carefully washed twice more with HBS to remove ASA. HUVEC were then isolated by collagenase/EDTA treatment, centrifuged (200g, 8 minutes, 22°C) and resuspended in incubation buffer. Centrifugation and resuspension were repeated twice to complete removal of serum-containing medium. Final EC suspensions were adjusted to  $10^7$  cells/mL and maintained at 22°C. These procedures yielded  $3 \times 10^6$  EC/75-cm<sup>2</sup> flask; EC suspensions had a viability of 75% to 90% (trypan blue exclusion). Radioimmunoassays for PGI<sub>2</sub> of stimulated HUVEC suspensions with or without ASA-treated platelets, verified inhibition of cyclooxygenase (data not shown).

**Platelet isolation.** Washed human platelet suspensions were prepared<sup>29,27,29</sup> using platelet-rich plasma (PRP) obtained from volunteers who had taken 650 mg ASA 12 hours before donating blood. To prepare  $^{14}\text{C}$ -5-HT-labeled platelets, 0.5  $\mu\text{Ci}$   $^{14}\text{C}$ -5-HT was added to each of four 50-mL centrifuge tubes containing 6 mL acid citrate dextrose (ACD) anticoagulant before blood collection. This resulted in complete ( $> 90\%$ ) uptake of  $^{14}\text{C}$ -5-HT by platelets during the blood collection process, determined by comparing the  $^{14}\text{C}$ -5-HT content of PRP and platelet-poor plasma (PPP; prepared by centrifugation of a 250- $\mu\text{L}$  aliquot of PRP, 15,000g, 1 minute, 22°C). Platelets were washed twice (4°C) in Tris-citrate buffer, resuspended in 0.154 mol/L NaCl, and adjusted to  $10^{10}$  platelets/mL. Before platelets were washed, complete inhibition of cyclooxygenase by ASA was verified in an aliquot of PRP by the absence of aggregation to 1 mmol/L sodium arachidonate.

**Aggregometry.** Platelet responses to agonists were recorded<sup>3</sup> in a Lumiaggregometer (Chronolog, Havertown, PA). A "platelet-poor" sample, containing all components except platelets, served

as control for 100% light transmission. To correct for light absorption by HUVEC suspensions, the platelet-poor cuvette contained a number of HUVEC equal to the "platelet-rich" cuvette.

Aliquots of platelets ( $58 \times 10^6$ ) and HUVEC ( $10^6$ ) were preincubated in combined suspension with stirring (1,000 rpm, 3 minutes, 37°C) in incubation buffer (final volume 350  $\mu\text{L}$ ) containing 0.7 mmol/L CaCl<sub>2</sub>. 5-HT-labeled platelets, with or without EC, were treated with 2.5  $\mu\text{mol/L}$  imipramine 1 minute before stimulation to block reuptake of released 5-HT.

After addition of agonist, platelet aggregation responses were recorded for 4 minutes. Changes in light transmission at 4 minutes were used to measure platelet aggregation. To arrest 5-HT secretion, cuvettes were then immediately placed on melting ice. Cuvette contents were centrifuged (3 minutes, 16,000g, 4°C) and secreted 5-HT was quantitated by scintillation counting of 50  $\mu\text{L}$  cell-free releasate.

In specified experiments, HUVEC were preincubated (3 to 5 minutes) with L-arginine and/or NMA or NNA before addition of platelets. Hemoglobin was added 40 seconds before thrombin or as specified to "intercept" and inactivate NO generated by EC. To inhibit platelet soluble guanylate cyclase, platelets were preincubated in stock suspension with 10  $\mu\text{mol/L}$  methylene blue (30 minutes, 22°C). An aliquot of treated platelets ( $58 \times 10^6$ ) was combined with EC for aggregometry experiments (final concentration of methylene blue, 0.3  $\mu\text{mol/L}$ ). As controls, untreated platelets and EC were preincubated in aggregometer cuvettes as usual, but with 0.3  $\mu\text{mol/L}$  methylene blue added.

In preliminary experiments, HUVEC cultured on microcarrier beads<sup>20</sup> interfered with our optical aggregometry measurements. In addition, some EC tended to detach from beads during stirring in aggregometer cuvettes, rendering use of microcarrier beads unfeasible in our experiments. We therefore investigated HUVEC in single cell suspension. Previously, we demonstrated biologic and biochemical interactions between platelets and such HUVEC suspensions at cell ratios (50 platelets per endothelial cell) closer to in vivo situations ( $\sim 1:1$ )<sup>3</sup> than possible when using EC in monolayer culture.<sup>3</sup> In the present studies, HUVEC in single-cell suspension and platelets were allowed to interact at a ratio of 58 platelets per endothelial cell.

**Fluid-phase transfer of HUVEC-derived EDRF/NO to platelets.** To ascertain that EC-dependent inhibition of platelet reactivity (measured as 5-HT release) was due to a fluid-phase component and did not require direct cell-cell contact (as do ADPases<sup>30</sup>), HUVEC and platelets were separated by a filter membrane using a "fluid-phase transfer system." This system was designed after attempts to separate EDRF-like activity from HUVEC by filtration through 0.45- $\mu\text{m}$  filters or by short centrifugation could not be physically accomplished. HUVEC suspensions were added to a Millicell-HA 12-mm culture plate insert (pore size 0.45  $\mu\text{m}$ ). The insert was placed in its original packaging, which was used as a container for the platelet suspension (Fig 1). Each compartment contained cells (EC and platelets, respectively) in a final volume of 250  $\mu\text{L}$  and was efficiently stirred at approximately 700 rpm. The combined fluid-phase transfer system was supported in a constant temperature bath (37°C) on a magnetic stirrer, allowing simultaneous or sequential addition of agonist (or drug) to each compartment independently (Fig 1). Imipramine was added to each compartment before addition of agonist. Four minutes after addition of agonist to both compartments, the complete transfer system was placed on ice to arrest 5-HT secretion. The contents of the platelet compartment were centrifuged (3 minutes, 16,000g, 4°C), and secreted 5-HT was quantitated by scintillation counting of 50  $\mu\text{L}$  cell-free releasate. Aggregation responses were observed qualitatively.

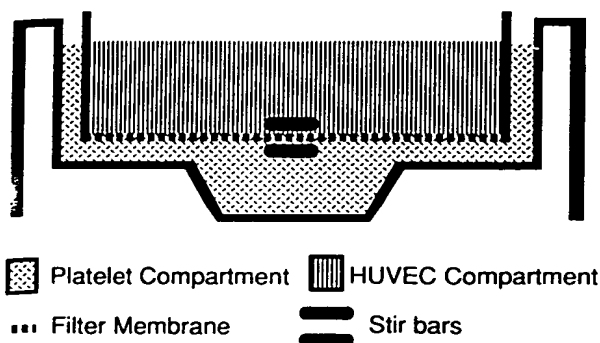


Fig 1. System for demonstration of fluid-phase transfer of EDRF, produced by HUVEC. A culture plate insert (filter membrane) separated EC and platelet suspensions. Upper and lower compartments contained  $10^4$  HUVEC and  $58 \times 10^4$  platelets, respectively, in a final volume of 250  $\mu$ L each, stirred at 700 rpm (37°C). After 4-minute preincubation and 4-minute thrombin stimulation, platelet serotonin secretion was measured by scintillation counting and aggregation was evaluated visually.

## RESULTS

*ASA-treated HUVEC suspensions inhibit aggregation of thrombin-stimulated, ASA-treated platelets through production of EDRF/NO.* In baseline dose-response experiments, in the absence of HUVEC, maximal aggregation of ASA-treated platelets was induced by 0.05 U/mL or more of thrombin. Baseline platelet responses to 0.03 U/mL thrombin varied from moderate to full and were donor-dependent (data not shown). With  $10^4$  ASA-treated EC added, platelet aggregation was completely blocked on stimulation of combined suspensions with 0.3 to 0.5 U/mL thrombin (Table 1). Therefore, ASA-treated EC completely inhibited platelet aggregation at levels of thrombin 10 times greater than those that fully aggregated platelets alone. Because  $PGI_2$  was not formed in these ASA-treated EC-platelet suspensions, as verified by radioimmunoassay (RIA), the inhibition demonstrated was independent of this eicosanoid. This ASA-insensitive blockade of platelet

Table 1. Endothelial Cells Inhibit Platelet Aggregation Upon Thrombin Stimulation of Combined Suspensions: Effects of EDRF Inhibitors

Additions	Aggregation (mm)
Platelets alone	123.42 $\pm$ 4.88 (n = 12)
Platelets + HUVEC	1.07 $\pm$ 4.14 (n = 15)
Platelets + HUVEC + Hb	122.58 $\pm$ 20.66 (n = 12)
Platelets + HUVEC + NMA	124.29 $\pm$ 26.09 (n = 7)
Platelets + HUVEC + NMA + arginine	46.00 $\pm$ 24.00 (n = 3)
Methylene blue-treated platelets + HUVEC	122.68 $\pm$ 18.65 (n = 5)

Values are means  $\pm$  SD of the extent of aggregation as measured by increased light transmission 4 minutes after addition of stimulus (0.3 U/mL thrombin), expressed as millimeters of deflection of aggregation curves. Hb 15  $\mu$ Mol/L was added 45 seconds before thrombin; NMA 1 mmol/L, with or without arginine 10 mmol/L, was preincubated with HUVEC (3 minutes, 37°C) before addition of platelets; platelets were incubated in stock suspension with methylene blue (10  $\mu$ Mol/L, 30 minutes, 22°C) before an aliquot was combined with HUVEC for aggregometry.

aggregation by EC was completely reversed by 15  $\mu$ Mol/L hemoglobin (Fig 2A and B, Table 1). In contrast, inhibition by EC was enhanced by 15 U/mL SOD. This was most effectively demonstrated in an experiment in which some aggregation in the absence of SOD did occur (Fig 2A). Platelet aggregation occurred with very similar slope and maximum extent whether hemoglobin was added immediately before or immediately after thrombin as well as when it was added 3 minutes after thrombin (Fig 2B).

EDRF/NO is derived from one of the guanidino nitrogens of L-arginine, as demonstrated with a specific arginine analogue NMA in cultured porcine EC.<sup>31,32</sup> In our system, 3 to 5 minutes preincubation of ASA-HUVEC with 1 mmol/L NMA reversed HUVEC-dependent inhibition of platelet aggregation (Fig 3, Table 1). This reversal was comparable to that attained with the EDRF scavenger hemoglobin. Addition of excess L-arginine (substrate for EDRF formation) to NMA before incubation prevented reversal of EC inhibition of platelet aggregation by NMA. Thus, L-arginine restored complete inhibition of platelet aggregation by ASA-treated HUVEC suspensions (Fig 3, Table 1). In contrast to NMA, the D-isomer was ineffective in prevention of EC inhibition of platelet aggregation (data not shown). Another competitive inhibitor of arginine metabolism, NNA, also reversed inhibition of platelet aggregation by HUVEC with an apparent  $IC_{50}$  of 20  $\mu$ Mol/L (data not shown).

EDRF/NO activates soluble guanylate cyclase,<sup>32,33</sup> an enzyme inhibitable by methylene blue.<sup>34</sup> Platelets pretreated in stock suspension with 10  $\mu$ Mol/L methylene blue (30 minutes, 22°C) before incubation with HUVEC aggregated on thrombin stimulation, in contrast to untreated platelets (Fig 4, Table 1). Extended pretreatment was required to demonstrate the methylene blue effect because its addition at 0.3  $\mu$ Mol/L final concentration during the 3-minute preincubation of EC-platelet mixtures before thrombin addition was ineffective (Fig 4). The action of methylene blue was not due to generation of superoxide (which destroys NO), because the results were essentially unaltered by SOD (Fig 4). These data demonstrate that HUVEC-derived EDRF/NO was acting through activation of platelet soluble guanylate cyclase.

*ASA-treated HUVEC suspensions inhibit 5-HT release by ASA-treated platelets through production of EDRF/NO.* 5-HT release was used as a parameter to measure effects of thrombin stimulation. With platelets alone, complete aggregation responses to thrombin (0.1 to 0.5 U/mL) were accompanied by 73.5%  $\pm$  10.9% to 94.2%  $\pm$  11.3% 5-HT release (data not shown). At 0.3 U/mL thrombin, the presence of HUVEC reduced platelet 5-HT release to 4.8%  $\pm$  4.3% (Table 2). Addition of SOD always augmented decreases in 5-HT release in the presence of HUVEC (data not shown). Removal of superoxide anion extended the half-life ( $t_{1/2}$ ) of NO, thereby enhancing HUVEC-dependent inhibition of platelet secretion as it did aggregation (Fig 2A). When combined suspensions of HUVEC and platelets were stimulated in the presence of hemoglobin, HUVEC-dependent inhibition was reversed and 5-HT secretion increased from 4.8% to 72.4% (Table 2). The

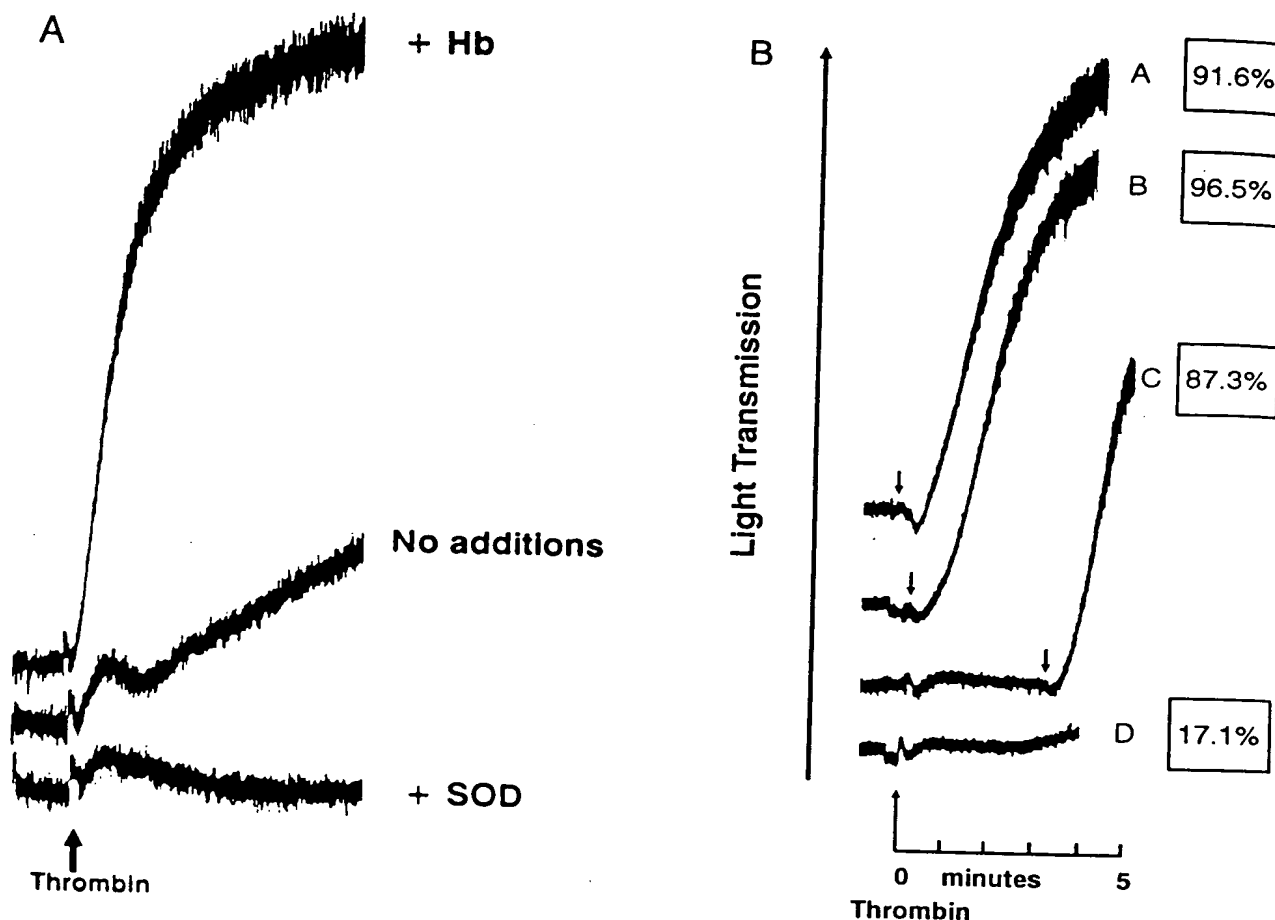


Fig 2. (A) Enhancement of HUVEC inhibition of platelet aggregation by SOD (15 U/ml), and reversal by 15  $\mu$ mol/L hemoglobin added 45 seconds before 0.3 U/mL thrombin. Aggregation curves are from a single experiment, representative of more than 20. (B) In separate experiments, 15  $\mu$ mol/L hemoglobin (arrows) reversed HUVEC inhibition of aggregation and 5-HT release (boxes), whether added 20 seconds before (curve A), 10 seconds after (curve B), or 3 minutes after (curve C) 0.5 U/mL thrombin. In controls without hemoglobin added (curve D), aggregation and 5-HT release remained inhibited. Aggregation curves are from a single experiment, representative of six.

effects of hemoglobin were similar whether added before or 10 seconds or 3 minutes after thrombin (Fig 2B).

Preincubation of HUVEC with NMA also resulted in an increase in 5-HT secretion (to 68.7%, Table 2). Similarly, platelet pretreatment with methylene blue reversed EC-dependent inhibition of 5-HT release (Fig 4). Thus, EDRF/NO modulation of platelet aggregation and dense granule secretion occurred in parallel.

*ASA-treated HUVEC suspensions inhibit 5-HT secretion by ASA-treated platelets in the absence of cell-cell contact.* EDRF/NO readily diffused from EC to platelets in a system devised to separate cells physically while permitting interaction through fluid-phase mediators (Fig 1). The presence of HUVEC reduced 5-HT secretion in the platelet compartment to 16.5% of control (Table 3). Control experiments demonstrated that molecules much larger than NO, such as thrombin, did not readily cross the membrane of the fluid-phase transfer system. Addition of hemoglobin to both compartments of the transfer system reversed inhibition of secretion (to 94.7% of control, Table 3). NMA or

NNA pretreatment of HUVEC had a similar effect and reversed inhibition of release to 85.4% of control (Table 3). The effects of NNA were blocked when a 10-fold excess of arginine was included during preincubation (Table 3), confirming that metabolism of a guanidino nitrogen of arginine was required to generate this fluid-phase inhibitor of platelet reactivity.

#### DISCUSSION

Our results demonstrate for the first time that single-cell suspensions of HUVEC block stimulated platelet 5-HT secretion and aggregation through generation of a short-lived, ASA-insensitive, fluid-phase reactant with the characteristics of EDRF/NO. Use of HUVEC suspensions allowed EC/platelet ratios of 1:58, which more closely approximate *in vivo* situations (up to 1:1 in the capillary circulation).<sup>3</sup> Such ratios are unattainable when EC monolayers are overlaid with platelet suspensions. In addition, cell-cell interactions between platelets and single cell suspensions of EC occur to a greater extent than do



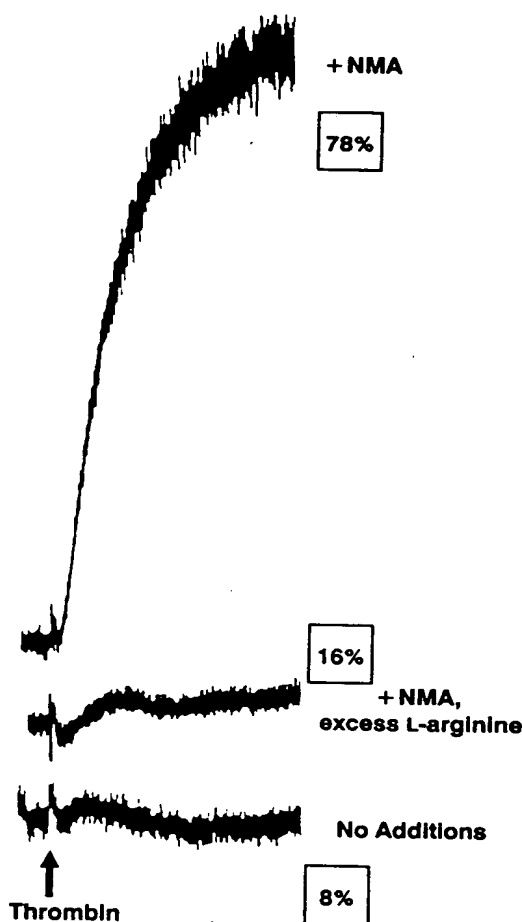


Fig 3. Reversal of EC-dependent inhibition of platelet aggregation and serotonin release (boxes) to 0.3 U/mL thrombin and requirement for metabolism of a guanidino nitrogen in L-arginine. HUVEC were preincubated with NMA (*N*<sup>ω</sup>-monomethyl-L-arginine acetate, 1 mmol/L, 3 minutes) before addition of platelets. The upper curve was obtained in 9 of 11 experiments. Preincubation of HUVEC with NMA with excess L-arginine added (10 mmol/L) restored inhibition of platelet responsiveness (representative of four experiments). Similar results were obtained with *N*<sup>ω</sup>-nitro-L-arginine methyl ester (NNA).

interactions between EC monolayers and platelets because of greater cell proximity.<sup>3</sup> Single-cell suspensions of EC also obviate the optical interference that occurs with use of polystyrene beads. Because EDRF/NO is a water- and lipid-soluble gas, directional (ie, apical v abluminal) secretion from EC is unlikely, even in vivo.

Effects of EDRF/NO on both platelet secretion and aggregation were prevented in three different ways: (1) by interruption of endothelial cell arginine metabolism (preincubation with the competitive substrate analogues, NMA or NNA), which blocked production of EDRF/NO by HUVEC and was overcome by provision of excess substrate, L-arginine; (2) by neutralization of EDRF/NO in transit between EC and platelets with hemoglobin and by extension of its t<sub>1/2</sub> with SOD; and (3) by pretreatment of platelets with methylene blue to block reactivity to

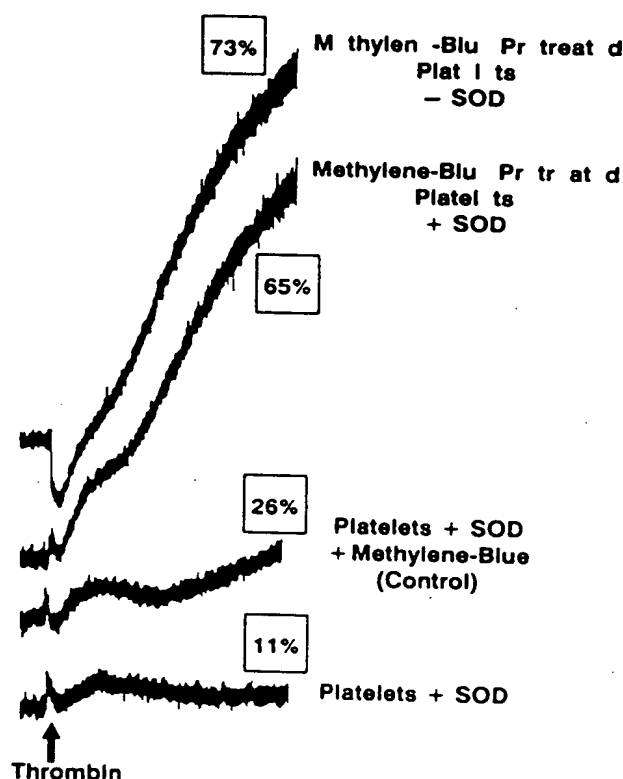


Fig 4. Reversal of EDRF effects by pretreatment of platelets with methylene blue (10  $\mu$ mol/L, 30 minutes, 22°C). HUVEC were unable to inhibit aggregation and 5-HT secretion (boxes) of pretreated platelets, induced by thrombin (0.3 U/mL), with or without SOD. Addition of methylene blue at a final concentration of 0.3  $\mu$ mol/L during preincubation and subsequent stimulation of combined suspensions did not affect EC-dependent inhibition of platelet responsiveness.

EDRF/NO through inhibition of platelet-soluble guanylate cyclase.

HUVEC produced fluid-phase EDRF/NO whether or not direct cell-cell contact occurred. Thus, platelet reactivity was blocked in both aggregometry experiments (Figs 2 through 4) and in the fluid-phase transfer system (Fig 1, Table 3). Several features distinguished this fluid-phase transfer system from other methods unsuccessful in yielding an EDRF-containing cell-free EC supernate. Effective mixing efficiently transferred a water-soluble gas (NO)

Table 2. Endothelial Cells Inhibit Serotonin Secretion by Thrombin-Stimulated Platelets: Effects of EDRF Inhibitors

Additions	Serotonin Secreted (%)
Platelets alone	81.0 $\pm$ 8.3
+ HUVEC	4.8 $\pm$ 4.3
+ HUVEC + Hb	72.4 $\pm$ 13.6
+ HUVEC + NMA	68.7 $\pm$ 11.5

Values are means  $\pm$  SD (n = 4 to 6) serotonin secreted by platelets (in aggregometer cuvettes) on stimulation with 0.3 U/mL thrombin, expressed as percentage of serotonin incorporated. Hb 15  $\mu$ mol/L was added 45 seconds before thrombin; NMA 1 mmol/L was preincubated with HUVEC for 3 minutes (37°C) before addition of platelets.

**Table 3. Endothelial Cells Inhibit Serotonin Secretion by Thrombin-Stimulated Platelets in a Fluid-Phase Transfer System: Effects of EDRF Inhibitors**

Additions	Serotonin Secreted (%)
Platelets alone	100
+ HUVEC	16.5 ± 9.8 (n = 5)
+ HUVEC + Hb	94.7 ± 5.7 (n = 4)
+ HUVEC + NMA/NNA	84.1 ± 16.5 (n = 4)
+ HUVEC + NNA + arginine	30.1 ± 8.7 (n = 3)

Serotonin secretion by thrombin-stimulated platelets was inhibited by a fluid-phase reactant (Fig 1) and was restored by inhibitors of EDRF formation. Data shown are means ± SD, expressed as percentage of controls (platelets alone). HUVEC and platelets (upper and lower compartments, respectively) were preincubated 4 minutes before addition of thrombin (0.3 U/mL) to each compartment. Hb 15 µmol/L was added to both compartments 45 seconds before thrombin. The arginine analogues NMA (1 mmol/L) or NNA (0.5 mmol/L) as well as arginine (5 mmol/L) were preincubated with HUVEC for 3 minutes (37°C) before platelets were added to the lower compartment (Fig 1).

from one compartment to the other across a membrane with 0.45-µm pore size. Thus, EDRF/NO ( $t_{1/2}$  3 to 5 seconds)<sup>35</sup> did not decay before exerting its inhibitory effect. Moreover, "degassing" of the NO at the air-filter interface (as with syringe-type filters) did not occur, thus preventing loss of the active principle from the cell-free supernate.

Hemoglobin was an effective tool with which to demonstrate the presence of EDRF/NO in stimulated HUVEC suspensions. The heme group of hemoglobin binds NO<sup>16,36,37</sup> and renders the NO unavailable for interaction with soluble guanylate cyclase in EDRF target tissues. In addition, hemoglobin solutions may enhance formation of superoxide anion, which is known to inactivate NO.<sup>20,38</sup> We noted that SOD enhanced HUVEC-dependent inhibition of platelet reactivity, which further supports the concept that EDRF/NO is involved in the inhibition.

The arginine analogues NMA and NNA blocked HUVEC inhibition of platelet reactivity in a dose-dependent manner (Fig 3). This effect was overcome by preincubation with excess L-arginine, demonstrating a requirement for an unblocked guanidino group in substrate L-arginine. Twice in 11 independent experiments, NMA pretreatment of HUVEC was ineffective; this may have resulted from variations in cell permeability for NMA in different EC preparations. Use of NMA to inhibit NO synthesis from L-arginine may also be subject to caveats, however, because HUVEC may metabolize NMA.<sup>39</sup> Demethylation of NMA<sup>39</sup> would result in increased intracellular levels of arginine, the substrate for NO synthesis.<sup>16,21</sup> In such instances, NMA would be an ineffective inhibitor of NO formation. NNA pretreatment of HUVEC was ineffective in only one of eight independent experiments. Enzymatic removal of the nitro substituent in NNA has not been reported.

Formation of EDRF/NO by human endothelium has implications for thrombosis as well as hemostasis. The short  $t_{1/2}$  of EDRF/NO limits its effects to the immediate microenvironment. This is further ensured by the inhibitory effects of hemoglobin,<sup>16,17,20,36</sup> (Fig 3). and, of note, by intact erythrocytes.<sup>40,41</sup> NO scavenging by erythrocytes as a consequence of their hemoglobin content would constitute a proaggregatory property of these cells. This is clearly

distinct from the enhancement of platelet reactivity by intact erythrocytes, but not erythrocyte lysates, as recently reported.<sup>42</sup> Thus, EDRF/NO represents an effective, localized mechanism for prevention of excessive platelet accumulation. This occurs by inhibition of both platelet secretion (5-HT release) and recruitment (aggregation). Concomitantly, local blood flow is enhanced by the vasodilatory action of EDRF on vascular smooth muscle. Our finding that platelet shape change is not affected by EDRF/NO (Figs 2 through 4) indicates that initial platelet activation does indeed occur. This strongly suggests that platelet hemostatic potential is preserved.

The data obtained when hemoglobin was added 10 seconds or 3 minutes after thrombin (Fig 2B) demonstrate that EC inhibition of platelet reactivity through EDRF/NO requires continued generation of EDRF/NO. The prompt initiation of both platelet aggregation and 5-HT release on hemoglobin addition, even 3 minutes after thrombin, also indicates that thrombin was not neutralized by HUVEC suspensions during incubation (Fig 2B, curve C). This observation was supported by theoretical considerations. The number of EC used ( $10^6$ ) furnish a maximum of only  $10^{11}$  molecules of thrombomodulin to serve as receptors for thrombin.<sup>43,44</sup> This is less than 10% of the number needed for neutralization of thrombin added (0.15 U/0.5 mL total volume).

The above data suggest that EDRF/NO is an important platelet inhibitory autacoid formed by "healthy" endothelium and is unaffected by aspirin treatment. In contrast, endothelium in atherosclerotic lesions may be deficient in EDRF production.<sup>45-47</sup> Our demonstration that HUVEC generate EDRF/NO in culture opens a new approach for further in vitro study of the control mechanisms governing formation of this autacoid.

The platelet inhibitory effects of EDRF/NO demonstrated in combined suspensions of ASA-treated platelets and ASA-treated HUVEC (Figs 2 through 4) provide evidence that fluid-phase antiaggregatory properties of EC are present in the complete absence of PGI<sub>2</sub> production. In addition, if cyclooxygenase is not inactivated by aspirin, the effects of EDRF actually synergize with those of PGI<sub>2</sub> through inhibition by cGMP of cAMP phosphodiesterase.<sup>49</sup> These data suggest that the direct inhibitory action of EC cyclooxygenase metabolites has been overestimated. Currently available therapeutic methods for thrombotic diatheses attributable to increased platelet reactivity are modest. Increasing EDRF/NO formation in the vasculature may enhance the platelet inhibitory potential of endothelium and would be a new approach to prevent or attenuate thrombosis.

#### ACKNOWLEDGMENT

We thank Corazone Delfin, and staff (Department of Obstetrics and Gynecology, Booth Memorial Medical Center, Flushing, NY) and Dr Janet Stein, Providencia Monchek, Barbara Walker, and Kum Mack (Department of Obstetrics and Gynecology, Beth Israel Medical Center, New York, NY) for cooperation in obtaining umbilical cords, Dr Katherine A. Hajjar for advice concerning HUVEC tissue culture techniques, and Jeffrey M. Sequeira and Lenore B. Safier for helpful discussions.

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